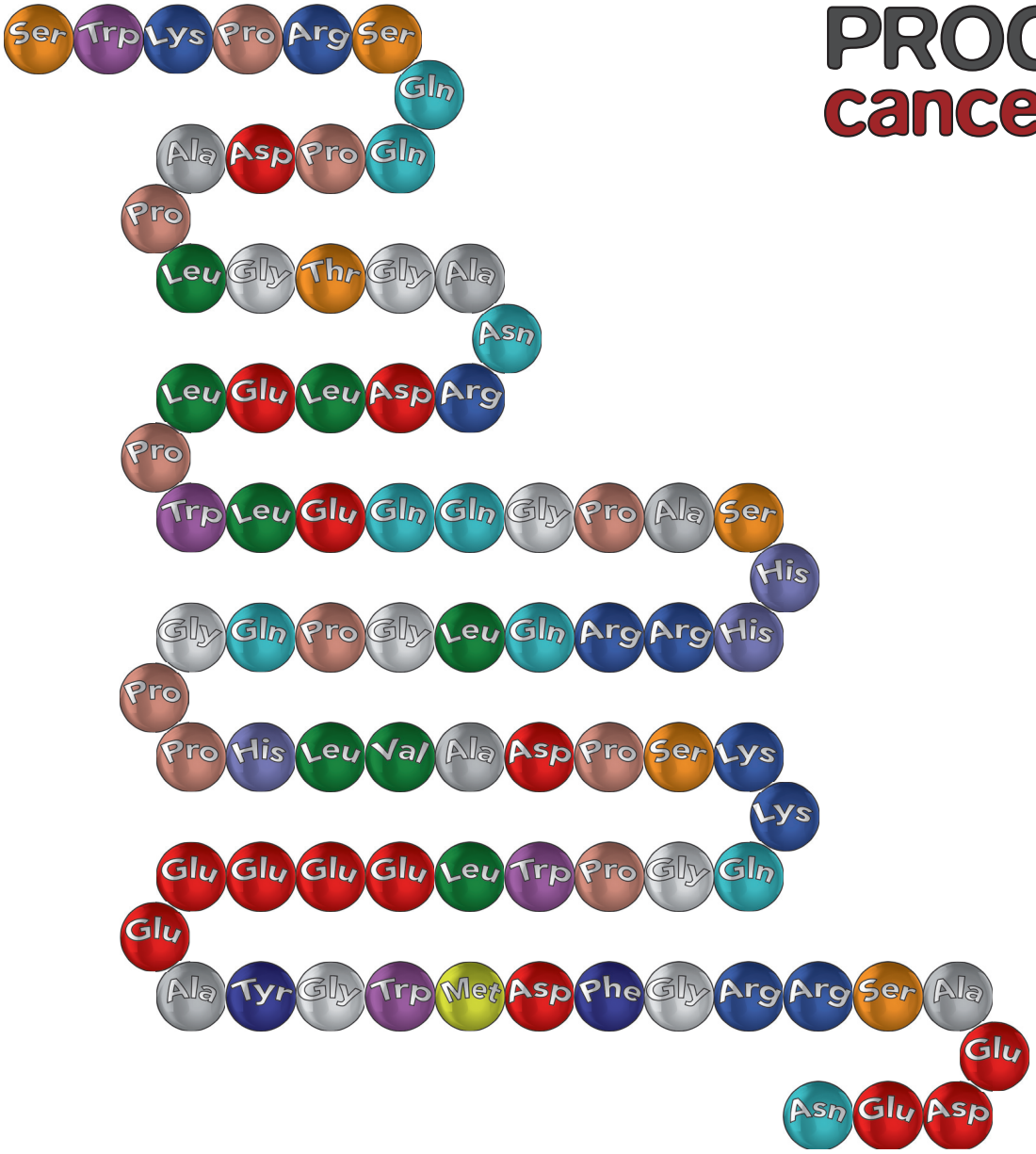

PROGASTRIN cancer control association

A review by
Dominique Joubert,
Ph.D. in biology,
and Alexandre Prieur,
Ph.D. in oncology

PROGASTRIN
AND ITS LINK TO CANCER:

WHITE PAPER
OCTOBER 2018

FROM THE DISCOVERY
OF PROGASTRIN
TO TUMOR REVERSION

PROGASTRIN AND ITS LINK TO CANCER:

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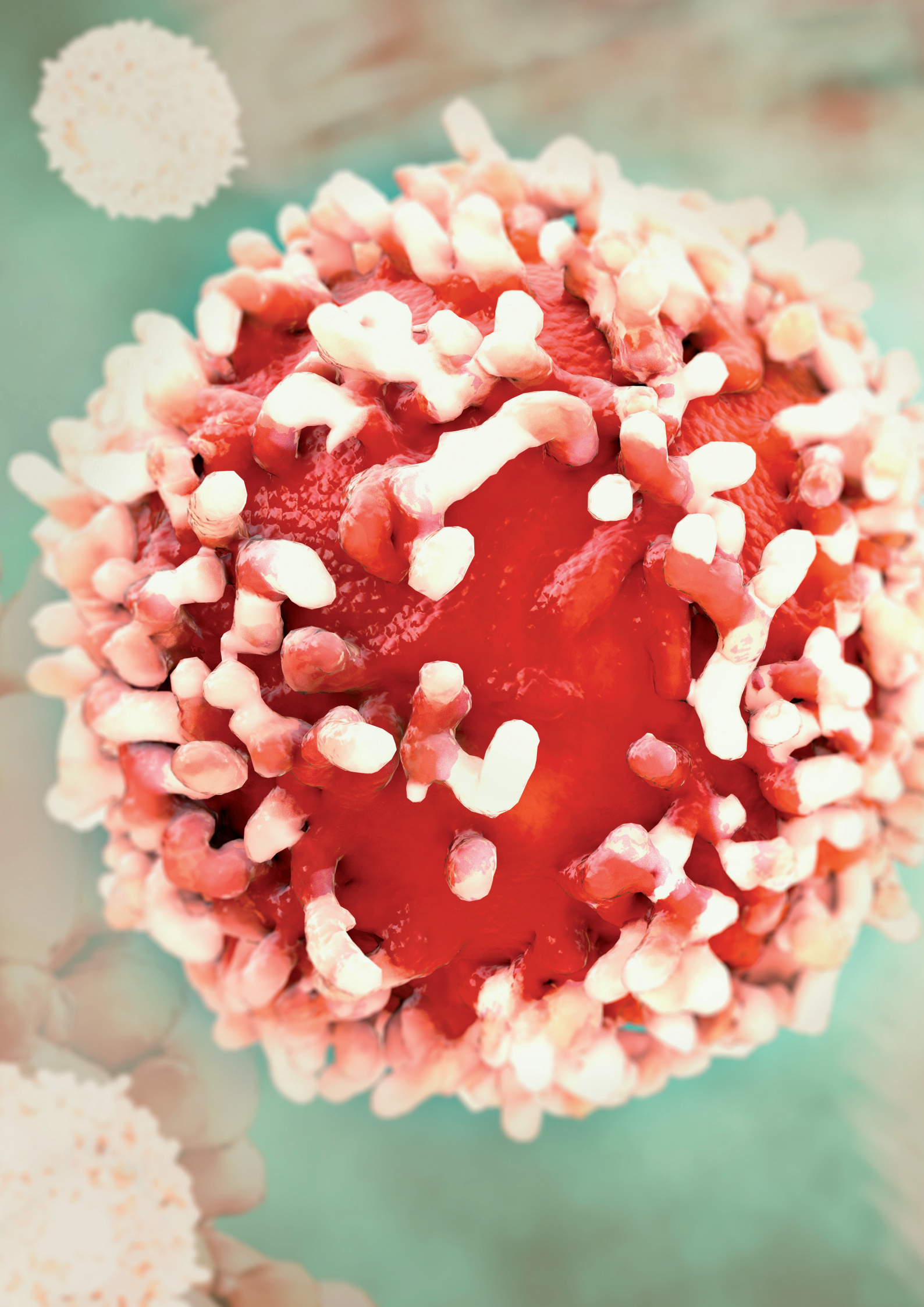


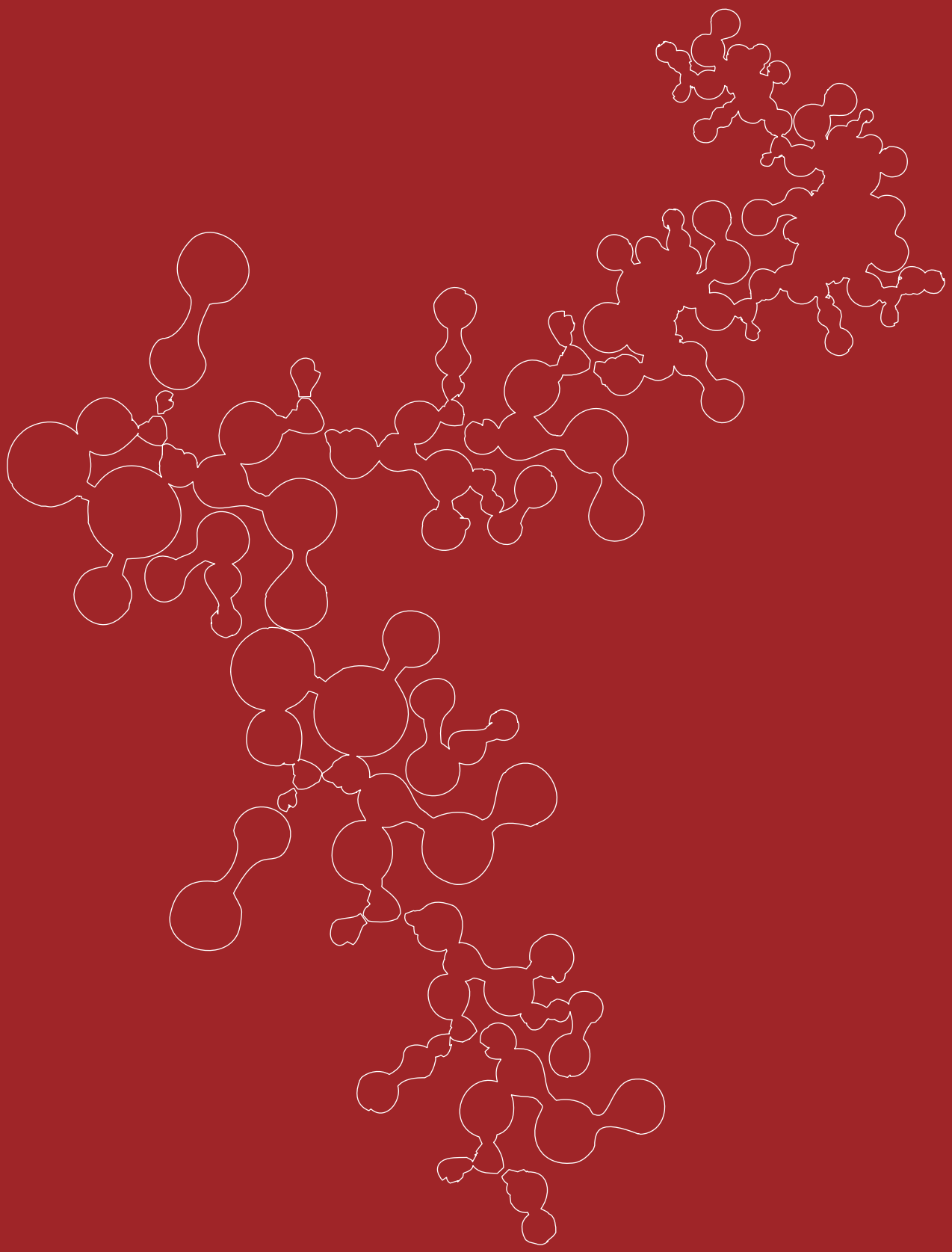
EDITO

PROGASTRIN,
A NEW TARGET IN THE FIGHT AGAINST CANCER

The work of many researchers around the world has demonstrated the multiple possibilities that progastrin offers in helping to detect and diagnose cancer, in supporting therapeutic follow-up, in monitoring relapses or in treating cancer itself, alone or in combination with other therapeutic means.

Today, the scientific knowledge gathered on progastrin and the mechanisms of its interaction with cancer are sufficiently demonstrated and solid to be made available to physicians, so that they can in turn define the best clinical methods for using and integrating these new means in their fight for patient health.

We asked Dominique Joubert Floch, Ph.D. in biology, and Alexandre Prieur, Ph.D. in oncology, to compile an objective and complete scientific review of the link between progastrin and cancer, which synthesizes and puts into perspective all the work accomplished, all the discoveries made, and the evidence collected by many laboratories since 1990.



CONTENTS

INTRODUCTION

This review will address a major issue: the role of progastrin in cancer. However, in order to go into this topic with a comprehensive understanding, we will first provide hereafter some general information, starting with this question: what is a tumor?

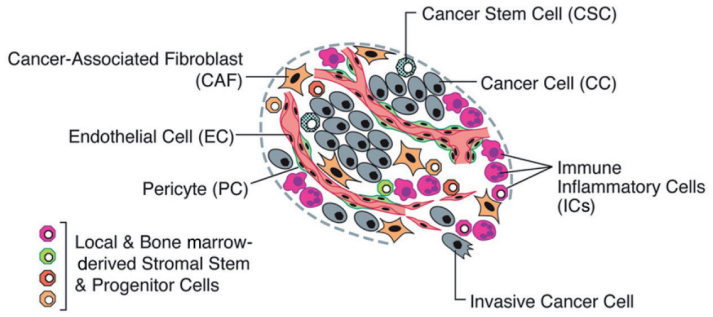


Figure 1
The complexity and heterogeneity of a tumor adapted from (Hanahan and Weinberg, 2011)

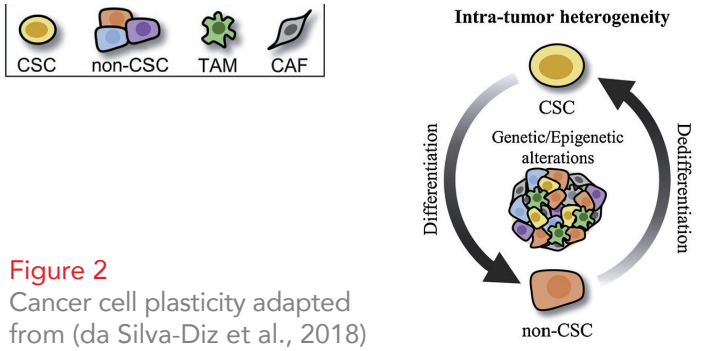


Figure 2
Cancer cell plasticity adapted from (da Silva-Diz et al., 2018)

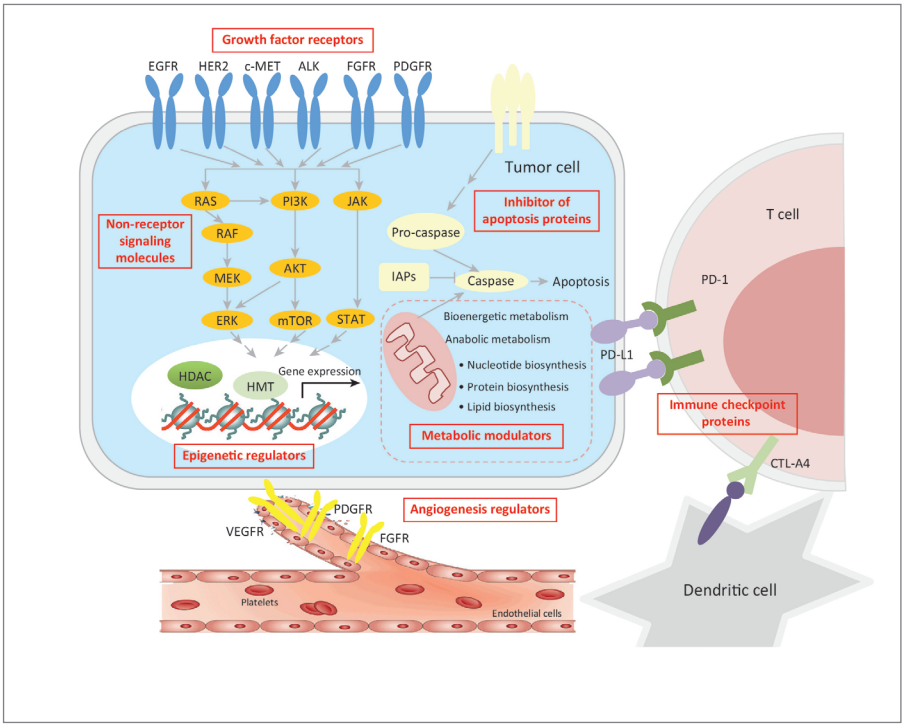
WHAT IS A TUMOR?

A tumor is a heterogeneous set of cells of which 1 to 5% have a cancer stem cell phenotype. These cells ensure the survival of the tumor and must therefore be the target of specific therapies (Kaur et al., 2018). They are capable of migrating and invading surrounding tissues, and of forming distant metastases. They are responsible for the generation of the cells that form the mass of the tumor: the progenitor cells which themselves will be able to enter a differentiation program, although often incomplete (Figure 1).

Tumor cells, like normal cells, do not have a stable phenotype (da Silva-Diz et al., 2018). This means that a progenitor cell, for example, could become a stem cell again if the tumor has an increased need for stem cells (Figure 2).

It is therefore crucial for tumor eradication to target both cancer stem cells and other cells. Today, the vast majority of therapies target proliferating cells, i.e. progenitor cells. This is the case with chemotherapy or therapies that target the mechanisms that ensure cell proliferation (Figure 3).

Furthermore, the growth of a tumor requires the formation of new vessels (neo-angiogenesis), in order to provide the tumor cells with growth factors and the oxygen necessary for their survival and proliferation. Cancer stem cells can survive in environmental conditions unfavorable to other cell types such as hypoxia

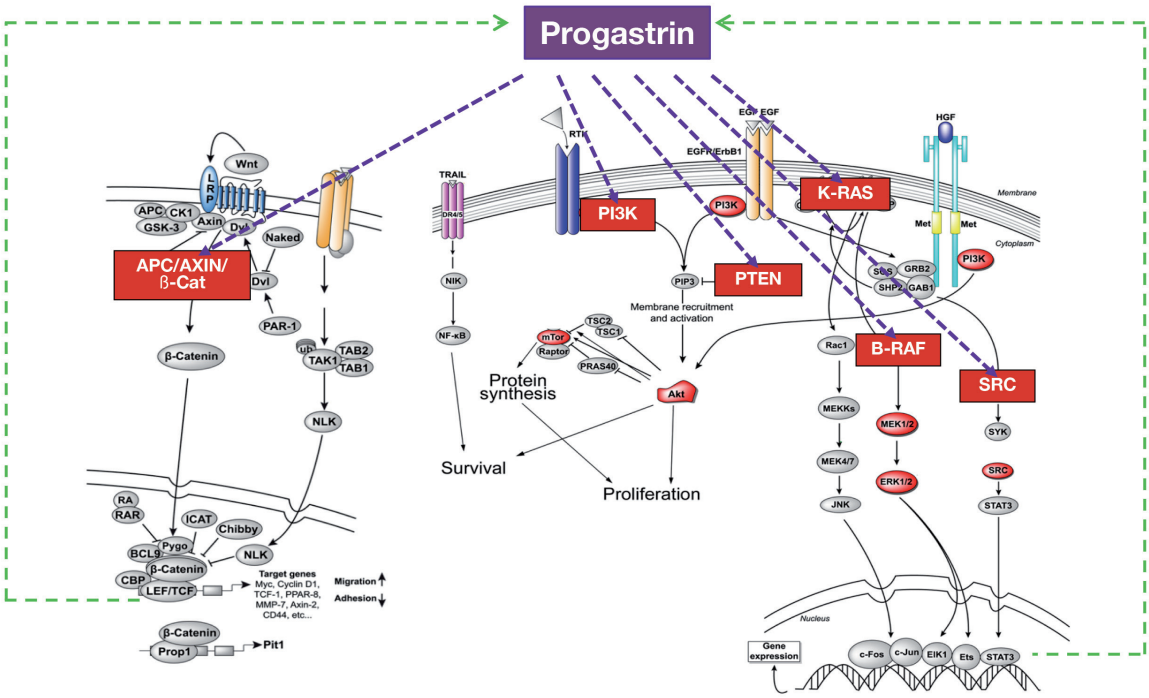


‣ Figure 3
Molecular target for targeted therapies adapted from (Huang et al., 2014).

or lack of growth factors. They can also survive chemotherapy treatments by using intracellular mechanisms capable of excluding chemotherapy molecules from the cell, making them resistant to these treatments (Batlle and Clevers, 2017).

The link between progastrin and cancer has been known for more than 30 years. Progastrin is involved in most of the properties of cancer cells that ensure the existence of the tumor: proliferation, survival of cancer stem cells in normoxia and hypoxia, cell migration and invasion, angiogenesis, and intracellular mechanisms responsible for the different properties of tumor cells (‣ Figure 4).

This document is an objective and comprehensive review of the link established by many laboratories between progastrin and cancer. We will analyze these links as we go along, starting with this question: what is progastrin? ◆



‣ Figure 4 Progastrin, a cornerstone of the oncogenic pathways adapted from (Hollande et al., 2010)

#01 WHAT IS PROGASTRIN?

In 1905, John Sydney Edkins showed the existence of a hormone responsible for gastric acid secretion. This hormone was called gastric secretin, or gastrin.

But it is only in 1979 (partial mRNA sequence: (Noyes et al., 1979)) and later on in 1987 and 1988 (human gastrin precursor: (Desmond et al., 1987; Dockray, 1988)) that progastrin was identified as the precursor of gastrin. Its sequence was unraveled as well as the sequence of its mRNA.

Progastrin is an 80-amino acid peptide, processed in the endoplasmic reticulum, with gastrin being the final active product of progastrin maturation.

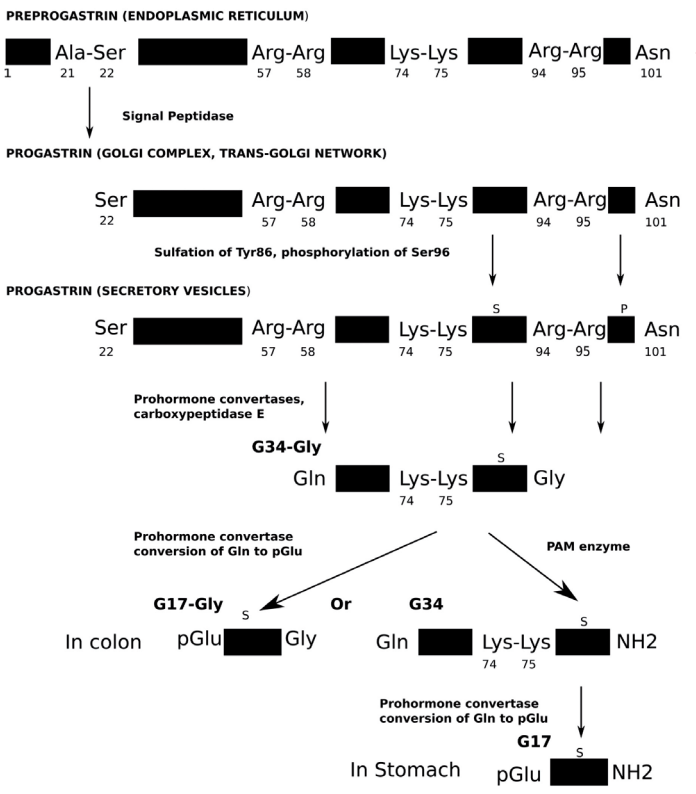
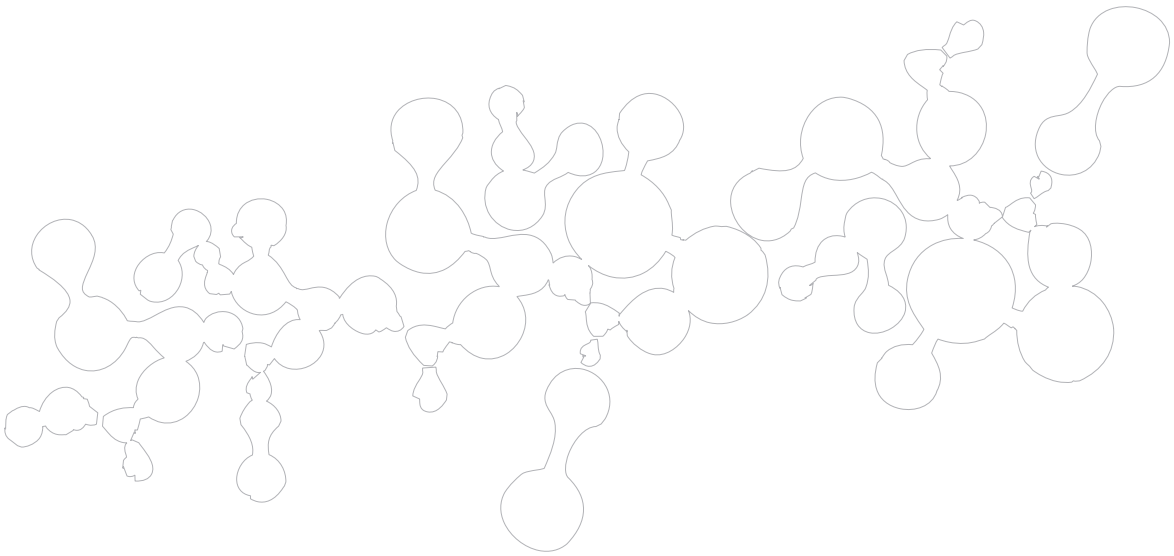


Figure 5
Processing of human progastrin adapted from (Copps et al., 2009)

Figure 5 shows how successive cleavages of progastrin end up with the final product gastrin (Copps et al., 2009). Sulfation and phosphorylation both play a role in the maturation process: they both increase processing of PG, while phosphorylation may also affect the conversion of glycine-extended gastrin intermediates (G34-Gly and G17-Gly) to mature gastrins (Bishop et al., 1998). ◆

#02

WHERE IS PROGASTRIN EXPRESSED IN PHYSIOLOGICAL CONDITIONS?



Progastrin is mainly expressed in the stomach, where gastrin is secreted from the G cells of the antrum. The major function of gastrin is to regulate acidic secretion.

The other maturation products, specifically G34-Gly, G17-Gly and CTFP (C Terminus Flanking Peptide), have been attributed various functions, and CTFP in particular has been described as being able to either induce or inhibit apoptosis, depending on the tissue or cell type concerned (Marshall et al., 2013; Patel et al., 2010; Smith et al., 2006)

Progastrin has been demonstrated to be expressed in other healthy tissue extracts also (cerebellum, pituitary, pancreas, testis), but to a much lesser extent than in the stomach, and the role that gastrin may have in these organs is not clearly understood (Bardram, 1990; Rehfeld, 1986; Rehfeld, 1991; Schalling et al., 1990). In the testis for instance, it is the carboxyamidated form of gastrin that are present in the sperm. The normal pancreas also expresses the gastrin mRNA, and it is hypothesized that the gastrinomas expressing progastrin originate from the progastrin-secreting pancreatic cells. ◆

#03

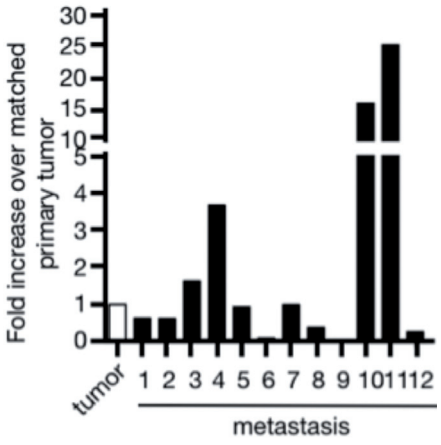
THE LINK BETWEEN PROGASTRIN AND CANCER: HOW WAS IT DEMONSTRATED?

Bardram was the first to hypothesize that "a low degree of processing of progastrin could serve as a predic-tor of a malignant clinical course at an early stage of the disease" (Bardram, 1990). He drew this conclusion after the evaluation of the presence of progastrin and its products in serum from Zollinger-Ellison patients (an endocrine disorder characterized by hyperproduction of gastrin due to a tumor (more often malignant than benign) or endocrine hyperplasia, most often located in the pancreas). He noted that the total progastrin product reflects tumor synthesis of gastrin better than conventional measurements of alpha-amidated gastrin.

After this observation, many publications described the presence of progastrin expression in cancers, starting with the first evidence from the use of a human gastrinoma where progastrin-derived peptides were purified and characterized (Huebner et al., 1991). However, progastrin was shown to be poorly processed in cancer cells, since processing enzymes are absent or not functional. This was clearly shown in colorectal cancer (Ciccotosto, 1995; Finley et al., 1993; Imdahl et al., 1995; Kochman et al., 1992; Nemeth et al., 1993; Singh, 1994; Van Solinge et al., 1993b). Indeed, Kochman showed that in colonic tissue, progastrin is over 700-fold more abundant than the amidated gastrin. In contrast, amidated gastrin in the human antrum is the predominant form of gastrin by a factor of 10. This was confirmed by Nemeth et al. using a different approach. Separation on Sephadex G50 revealed that most colorectal carci-nomas contain peptides derived from the gastrin precursor, progastrin, but for the most part these tumors do not convert progastrin into biologically active products. Immunostaining also showed that in a series of 23 adenocarcinomas, more than 50% of tumor cells stained for gastrin and progastrin. Then, Singh et al. (1994) demonstrated that progastrin was incompletely processed in human colon cancer cell lines and, more impor-tantly, was secreted from these cells cultured *in vitro*, opening the door to the analysis of a functional autocrine/paracrine function of progastrin in tumor cells ((Singh et al, 1994; Van Solinge et al., 1993b) and ‣Figure 6). Colorectal cancers are not the only cancer type to express progastrin. Ovarian cancers do as well, although concentrations of progastrin are much lower than those of amidated gastrin (van Solinge et al., 1993a), and liver tumors express precursor forms of gastrin, progastrin in particular, unlike normal liver (Caplin et al., 1999). Pancreatic tumors also express the gastrin gene, with 91% of the tumors secreting the unprocessed progastrin product (Caplin, 2002). Thus, progastrin is expressed in various types of tumors, and is secreted from cancer cells *in vitro*.

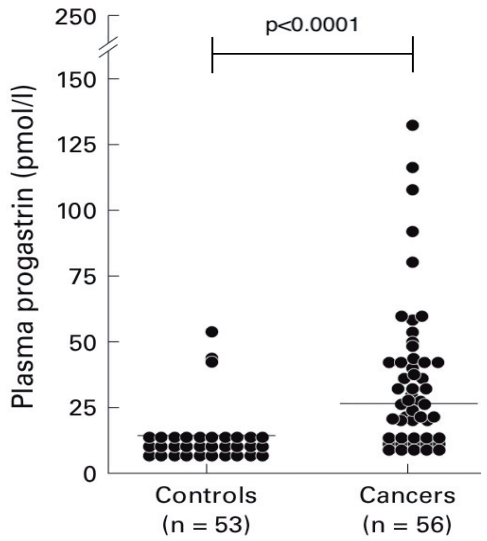
Therefore, the next question is: Can we detect progastrin in the blood of cancer patients?◆

‣Figure 6
Progastrin is expressed in human colorectal cancer. Progastrin expression was analyzed in the primary tumors of 12 patients and in the metastases present in the same patients. Results are shown as fold increase of the expression in the metastasis over matched primary tumor.



Thus, **progastrin is expressed in various types of tumors, and is secreted from cancer cells *in vitro*.**

#04 PROGASTRIN IS PRESENT



‣ Figure 7
Progastrin is present in the plasma of colorectal cancer patients. Progastrin was assayed in the plasma of colorectal cancer patients and in a series of controls using radio immunoassays. Colorectal cancer patients had a significantly higher concentration of progastrin than controls (adapted from (Siddheshwar et al., 2000)).

The evidence showing that progastrin could be detected and quantified in the blood of colorectal cancer patients was demonstrated by Siddheshwar et al. as early as 2000 ((Siddheshwar et al., 2000) and ‣ Figure 7).

These authors provided clear-cut data on the increase of plasma progastrin levels and not amidated gastrin in colorectal cancer patients compared to a series of controls. They also studied a series of patients with adenomatous polyps and observed an increase of progastrin, even though this increase was not statistically significant, unlike what was observed by Prieur et al. in 2017 (Prieur et al., 2017). In this later work, progas-trin was assayed with a very sensitive sandwich Elisa test. This technical approach allowed a much better detection of the increased level of blood progastrin in 67% of patients with adenomatous polyps.

Interestingly, Siddheshwar et al. had also shown that fasting plasma total gastrin levels were similarly increased in the blood of colorectal cancer patients whatever the status of Helicobacter pylori (positive or negative). This was due to unprocessed gastrins since amidated gastrin levels were unchanged.

IN THE PLASMA OF COLORECTAL CANCER PATIENTS

Thus, progastrin is present in the tumor and in the blood of colorectal cancer patients. But does tumor progastrin accounts for all blood progastrin?

This was demonstrated by Konturek et al. in 2002 (Konturek et al., 2002). These authors measured progas-trin in the blood of colorectal cancer patients before and after surgery. Levels were increased as expected in colorectal cancer patients compared to controls before surgery and went back to normal values after surgery.

All the arguments were therefore available to the scientific community to start analyzing the function(s) of progastrin in tumor cells. The rationale was present, and as you will see below, the results indeed demonstrated the major role that progastrin exerts on the tumor, providing the soil today for considering progastrin as a new cancer target in the fight against cancer. ◆

These authors provided clear-cut data on the **increase of plasma progastrin levels and not amidated gastrin in colorectal cancer patients compared to a series of controls.**

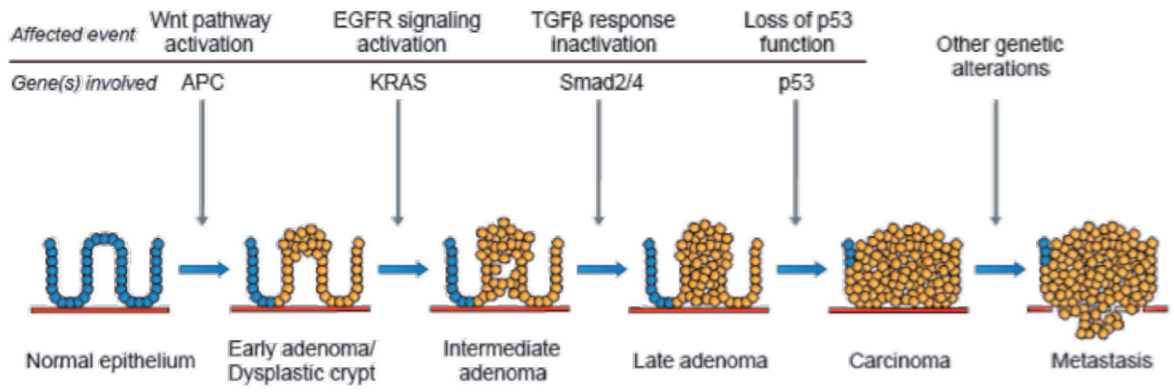
#05

PROGASTRIN AND ITS FUNCTIONS IN CANCER CELLS

In order to understand how important, the role of progastrin in cancer cell regulation is, it is crucial to understand how a tumor is initiated and progresses. And the best model to do so is the colorectal cancer model.

Colon tumorigenesis initiates some 30 years before colon cancer becomes symptomatic. The first event leading to colon cancer is the constitutive activation of the wnt/ß-catenin oncogenic pathway induced by the mutation of either APC (the most frequent case) or the ß-catenin gene. It has been demonstrated that the introduction of these mutations in normal stem cells of the intestine is indeed sufficient to initiate tumorigenesis (Huels and Sansom, 2015). These mutations induce the formation of an adenoma with preneo-plastic features, followed by the evolution towards an adenocarcinoma. Then, other mutations occur lead-ing to the activation of other oncogenic signaling pathways (‣ Figure 8). In order to reach this stage of tumor development, cells have to proliferate, and become independent from their neighboring cells (this is called loss contact inhibition). Then, tumor cells have to acquire an EMT (Epithelial-Mesenchymal Transition) phenotype in order to invade adjacent normal tissue and eventually form distant metastases. In addition, for the tumor to grow, new blood vessels have to be generated (via neo-angiogenesis), and cells have to evade immune surveillance in order to escape T-cell recognition.
All these features are controlled by intracellular mechanisms, among which are certain signaling pathways of paramount importance.

We will now provide evidence from the literature demonstrating that progastrin is involved in the majority of the mechanisms, tumor cells use to survive and grow.



‣ Figure 8
Schematic representation of intestinal tumorigenesis, showing accumulation of mutations and activation of signaling pathways (adapted from Syscol-project)

As early as 1996, **gastrin gene expression was shown to be required for human colon cancer cell tumorigenicity.** (Singh et al., 1996)

PROGASTRIN AND TUMOR CELL PROLIFERATION

As early as 1996, gastrin gene expression was shown to be required for human colon cancer cell tumorigenicity. (Singh et al., 1996)

Singh and co-authors investigated the functional role of the gastrin gene by examining the effect of gastrin antisense (AS) RNA expression (resulting in progastrin production inhibition) on the growth and tumorigenicity of colon cancer cells. The proliferative and tumorigenic potential of the AS clones from the gastrin-expressing cell lines was significantly suppressed compared to that of the control clones. From these observations, authors anticipated that the growth of a significant percentage of colorectal cancers may be critically dependent on the expression of gastrin gene products.

Among the maturation products of progastrin, glycine-extended gastrin may also play a trophic role in tumorigenesis. Indeed, Hollande et al. in 1997 (Hollande et al., 1997) showed that glycine-extended gastrin acts as a trophic factor in non-transformed cells, and Stepan et al. in 1999 (Stepan et al., 1999) showed that glycine-extended gastrin stimulated the growth of HEK cells and of human colon cancer cells *in vitro*.

PROGASTRIN IS A PROMOTER OF INTESTINAL TUMORIGENESIS

To demonstrate the role of progastrin in intestinal tumorigenesis *in vivo*, two mice models were generated: one was gastrin gene deficient, and the other overexpressed the gastrin gene.

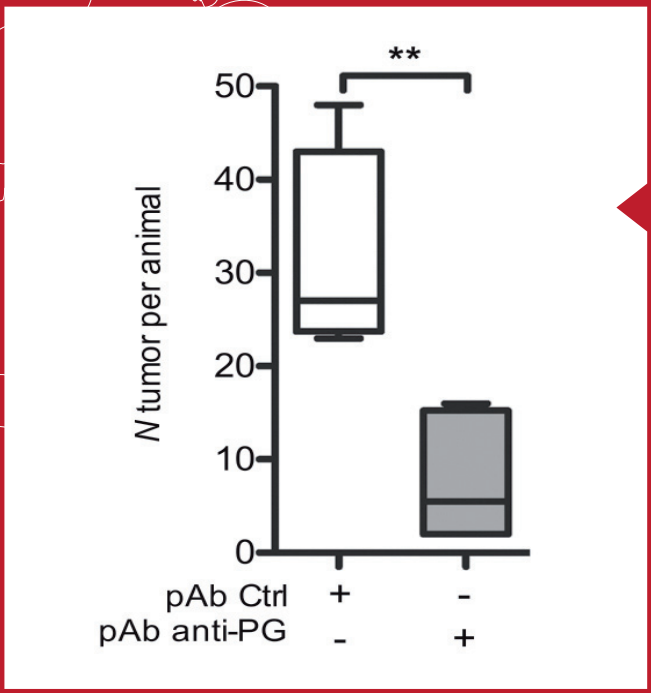
Koh et al. in 1997 generated gastrin deficient mice (Koh et al., 1997). They observed that the colon was histologically normal, indicating that neither progastrin nor gastrin play a major role in the physiology of the colon.

Then, transgenic mice overexpressing human gastrin gene without targeting a specific tissue (hGAS) or as an insulin gastrin transgene (INS-GAS) were generated (Wang et al., 1996). The pancreatic islets of INS-GAS mice were able to produce carboxyamidated G-17, resulting in a twofold elevation of serum amidated gastrin, marked thickening of the oxyntic mucosa, and an increased proliferation index of the gastric body. In contrast, livers of adult hGAS mice expressed abundant human gastrin mRNA and human progastrin but were unable to process this peptide to the mature amidated form, resulting in markedly elevated serum progastrin levels and normal amidated gastrin levels. These mice had an increased proliferation index in the colon suggesting that incompletely processed gastrin precursors may contribute to colonic mucosal proliferation *in vivo*. The overexpression of glycine-extended gastrin in transgenic mice was also shown to result in increased colonic proliferation (Koh et al., 1997).

In these genetically modified mice, colonic proliferation was increased but did not result in the formation of a tumor. Clearly, progastrin was not a tumor initiator. The proof that progastrin could be a tumor promoter was then obtained in mice predisposed to tumor development. Two experimental set-ups were used:

1. mice overexpressing progastrin were treated with azoxymethane (AOM), a chemical carcinogen, (Cobb et al., 2004; Singh, 2000; Singh et al., 2000), leading to a significant increase of tumor formation.
2. mice bearing a mutation in the APC gene $APC^{min/+}$ were crossed with gastrin-deficient mice (Koh et al., 2000). In the $APC^{min/+}$ mice, one allele of the APC gene is mutated leading to its inactivation, therefore each time a cell loses the second allele of APC, the Wnt/ß-catenin pathway is constitutively activated, initiating intestinal tumorigenesis, first with spontaneous adenomas and then formation of adenocarcinomas. In gastrin-deficient $APC^{min/+}$ mice, there was a marked decrease in polyp numbers and a significantly decreased polyp proliferation rate.

Pannequin et al. in 2007 (Pannequin et al., 2007) and Prieur et al. in 2017 (Prieur et al., 2017) used another mouse model bearing a different mutation in the APC gene called the $APC^{\Delta 14/+}$ mouse model. These mice, as the $APC^{min/+}$, spontaneously develop adenomas and adenocarcinomas, but with a higher number of these tumors in the colon. In both papers, progastrin was impaired by treating the mice with an siRNA (Pannequin et al., 2007) or with a neutralizing anti-progastrin antibody (Prieur et al., 2017). Interestingly, as with $APC^{min/+}$ mice, in all the cases, inhibiting or neutralizing progastrin leads to a decrease in the number of tumors (‣ Figure 9).



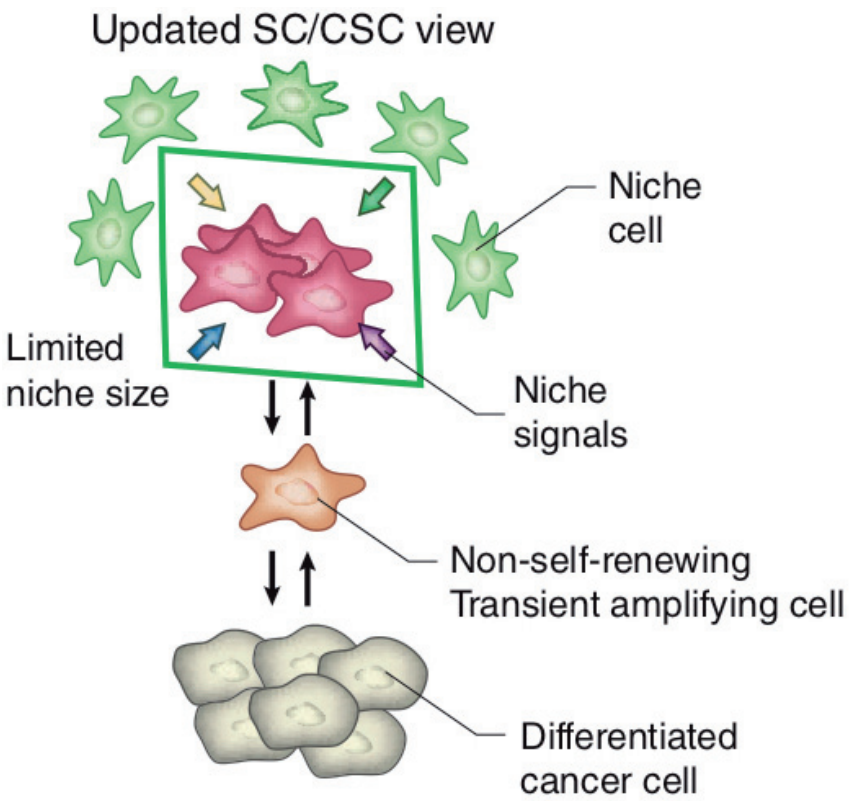
‣ Figure 9
Number of tumors in the intestinal tract of $APC^{\Delta 14/+}$ mice treated with control or anti-progastrin antibody (adapted from (Prieur et al., 2017))

Since it is essentially progastrin that is secreted by the tumor cells and not the maturated products, **progastrin may therefore represent an early event in colorectal tumorigenesis and may contribute significantly to tumor progression.**

These results highlight the role of progastrin as a tumor promoter. Since it is essentially progastrin that is secreted by the tumor cells and not the maturated products, progastrin may therefore represent an early event in colorectal tumorigenesis and may contribute significantly to tumor progression.

PROGASTRIN IS ESSENTIAL FOR CANCER STEM CELL SURVIVAL

Cancer stem cells represent a small proportion, supposedly between 1 and 5%, of the tumor. But they are mostly important for the survival of the tumor as they play the role of a "reactor". Without them, the tumor does not survive. They have the capacity to self-renew and to generate all the other cell types present in the tumor by asymmetric division, starting with progenitors that have a high propensity to proliferate (‣ Figure 10).



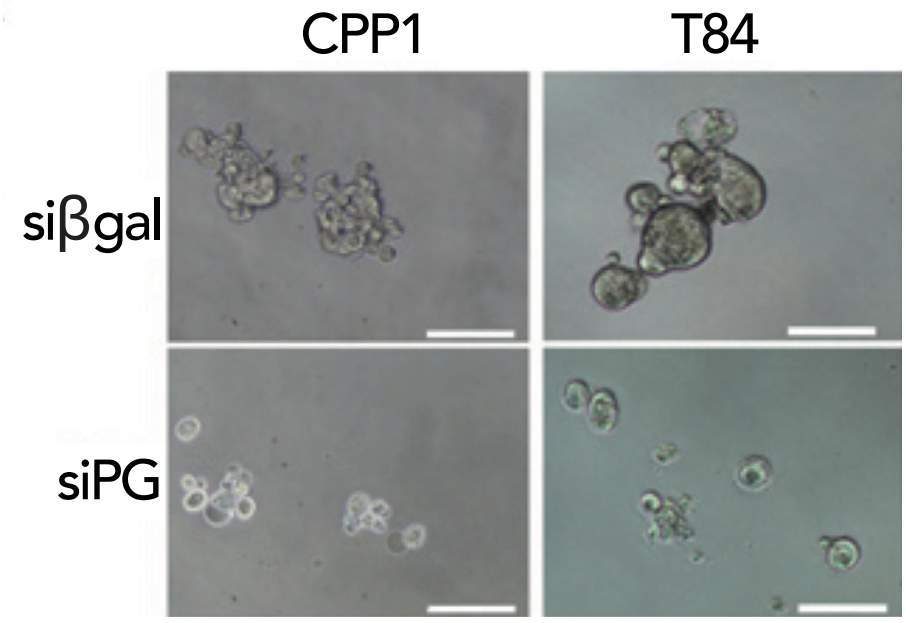
‣ Figure 10
Features of cancer stem cells (in red on the scheme) hierarchies adapted from (Batlle and Clevers, 2017)

Cancer stem cells do not proliferate at a high rate, and therefore escape treatments that target proliferating cells, such as chemotherapy. They can migrate and invade surrounding tissues and are thus at the origin of distant metastases (Batlle and Clevers, 2017).

Whereas it is crucial to target cancer stem cells, it cannot be done without also targeting the other cells. Indeed, phenotypes are "plastic" and a progenitor can go back to a cancer stem cell phenotype if there is an increased need for cancer stem cells (‣ Figure 2).

It was therefore important to understand if the *in vivo* effect of progastrin in intestinal tumorigenesis involved the regulation of cancer stem cells. Such a role was suggested from the observation that progastrin was expressed in CD133-positive colorectal cancer cells, which express some of the phenotypic features of cancer stem cells (Ferrand et al., 2009).

It is however Giraud and co-authors who really demonstrated the major role that progastrin plays in can-cer stem cells (Giraud et al., 2016). They first showed that progastrin expression, both at the mRNA and protein levels, was highly increased in colorectal cancer cells cultured in conditions where cancer stem cells are enriched (non-adherent conditions, sphere forming assay). Progastrin was then showed to be mandatory for the formation of spheres that require a cancer stem cell to start growing. This indicated that progastrin could regulate cancer stem cell frequency, which was subsequently demonstrated *in vitro* as well as *in vivo* (‣ Figure 11).



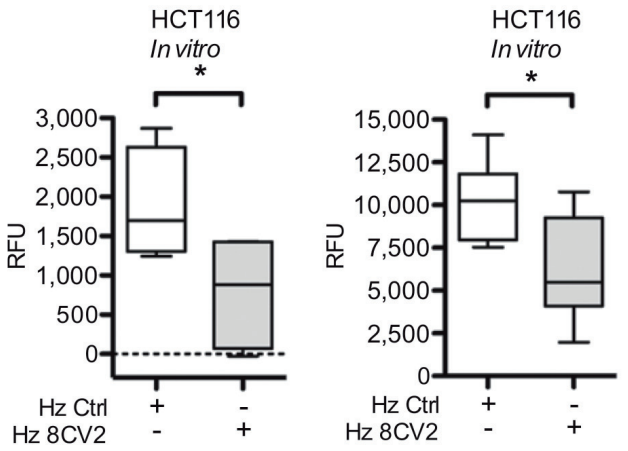
‣ Figure 11
Progastrin depletion impairs CSC survival and self-renewal *in vitro*
adapted from (Giraud et al., 2016)

These two papers clearly demonstrate that **progastrin is a survival factor for colorectal cancer stem cells**.

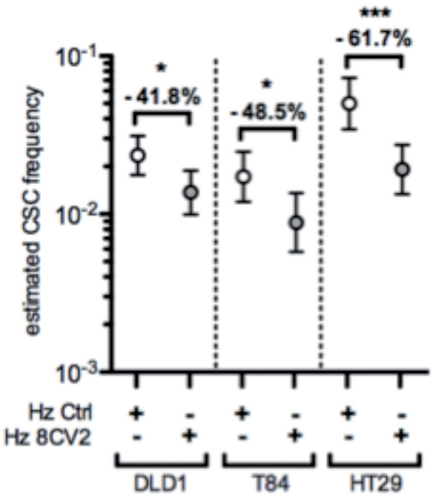
Later, it was also shown by Prieur et al. that migration and invasion, two characteristics of cancer stem cells, are both greatly affected *in vitro* and *in vivo* (‣ Figure 12).

Furthermore, it is the secreted progastrin that plays the role of a cancer stem cell survival factor. Indeed, when a neutralizing antibody is added to the culture or when mice engrafted with human colorectal cancer cells are treated *in vivo* with such an antibody, the frequency of cancer stem cells is similarly decreased ((Prieur et al., 2017) and ‣ Figure 13).

These two papers clearly demonstrate that progastrin is a survival factor for colorectal cancer stem cells.
Thus, progastrin might enable the targeting of the tumor's engine and, consequently, the tumor itself. ◆



‣ Figure 12
Anti-progastrin antibody inhibits migration (A) and invasion (B) of colorectal cancer cells (adapted from (Prieur et al., 2017))



‣ Figure 13
Anti-progastrin antibody decreases CSC frequency of colorectal cancer cells (adapted from (Prieur et al., 2017))

#06

BY WHICH MECHANISMS DOES PROGASTRIN CONTROL TUMOR FORMATION?

PROGASTRIN DECREASES APOPTOSIS

As we already discussed, progastrin can stimulate tumor cell proliferation. Wu et al. showed that it is also able to reduce apoptosis (Wu et al., 2003). This was demonstrated in gastrin-responsive intestinal epithelial cells cultured in the presence of progastrin. A significant loss in the activation of caspases 9 and 3, resulting in a significant loss in DNA fragmentation upon PG treatment of the cells was observed.

Therefore, the effect of progastrin on cell survival results both from an increase in proliferation and from a decrease of apoptosis.

PROGASTRIN REGULATES ADHERENS AND TIGHT JUNCTIONS

For a cell to proliferate and migrate, it must become independent from its neighboring cells. The integrity of cell-cell contacts is essential for the prevention of metastasis formation, which first requires cell migration. Hollande et al. in 2003 (Hollande et al., 2003) demonstrated the major role that progastrin plays on both adherent and tight junctions between neighboring cells. In progastrin-secreting DLD-1 human colorectal carcinoma cells, expression of an antisense gastrin construct restored membrane localization of proteins constitutive of these junctions (zonula occludens-1 (ZO-1), occludin, ß-catenin and E-cadherin). This effect involved both enhancement of Src tyrosine kinase activity and induction of a spatial delocalization of protein kinase Cα (Hollande et al., 2003).

PROGASTRIN IS A PRO-ANGIOGENIC FACTOR

When a tumor grows, it requires additional oxygen and nutrients provided by new blood vessels. The gen-eration of new blood vessels is called neo-angiogenesis. Recently, in 2014, progastrin was shown to be a pro-angiogenic factor, meaning that it induces the formation of blood vessels (Najib et al., 2014). Progastrin stimulated endothelial cell proliferation and migration and increased the ability of endothelial cells to form capillary-like structures in vitro. *In vivo*, when progastrin production was blocked by shRNA in cells xenografted in nude mice, neovascularization of the tumor was decreased. These observations, coupled to a mechanistic understanding at the level of vascular endothelial-cadherin, p125-FAK and paxillin, provided the necessary evidence for the demonstration of the role of progastrin as a pro-angiogenic factor.

PROGASTRIN AND HYPOXIA

A tumor is not a homogeneous set of cells because there are areas, mostly in the middle of the tumor, that are less vascularized than the remaining part of the tumor. In these specific regions, hypoxic conditions are therefore a constraint for the cells that are present.

Cancer stem cells have adapted to resist to hypoxic conditions. They can survive in this stringent environment where other cell types die.

The first evidence of a link between progastrin and hypoxia was provided by the work of Najib et al. who showed that *in vivo*, overexpression of progastrin provides a physiological advantage to mice under hypoxic conditions (Najib et al., 2014). Later, in 2017, Prieur et al. have shown that *in vitro*, progastrin expression is boosted in hypoxic conditions, which is in line with the fact that cancer stem cells express higher levels of progastrin than the other tumor cells (Prieur et al., 2017). Progastrin might thus help cancer stem cells survive in hypoxic conditions.

Therefore, progastrin, via a variety of mechanisms that are all crucial for tumor growth and survival, can be considered as a major tumor promoter. Its major function is to help cancer stem cells survive and spread in order to form metastases, which is probably why progastrin can also be considered as a potential predictive marker of liver metastasis in colorectal cancer (Westwood et al., 2017).

The questions now are:

- **How is progastrin able to exert these functions?**
- **What is the receptor of progastrin that transduces its signal?**
- **What are the intracellular mechanisms involved?**
- **Does progastrin have a direct link with oncogenes?** ◆

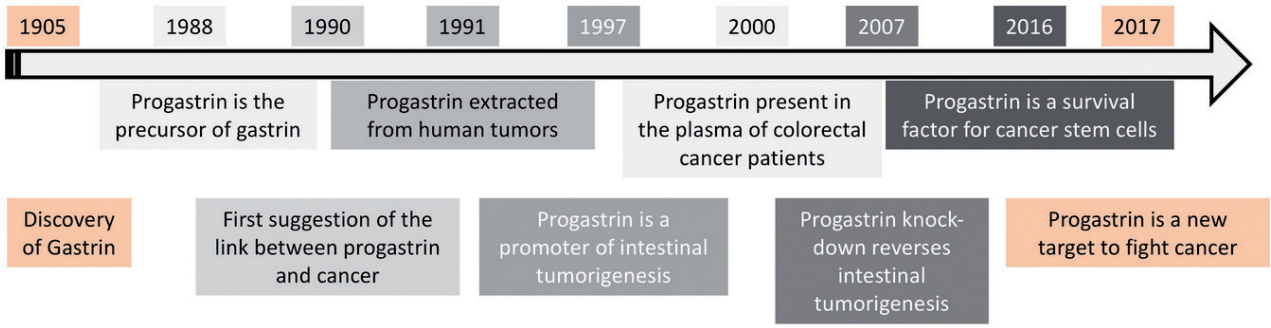


Figure 14
Timeline of the major dates that describe the link between progastrin and cancer, from the discovery of gastrin to the demonstration that progastrin is a new target in the fight against cancer.

#07

WHAT IS THE RECEPTOR OF PROGASTRIN?

Despite continuous efforts deployed by the scientific community to identify and characterize the progastrin receptor, it is not yet clearly identified. We will now review all the candidates and try to understand why they are unlikely to be the true progastrin receptor.

WHAT IS CERTAIN: THE RECEPTOR EXISTS

High affinity binding sites were first described in IEC cells using iodinated recombinant human progastrin. Affinity was in the order of 0.5-1 nM, which was compatible with a receptor. When biotinylated progastrin binding to cells was assessed using flow cytometry, a strong and specific binding of progastrin to some cell lines (IEC-6, IEC-18, HT-29, COLO320) was also detected (Dubeykovskiy et al., 2008). The specificity of binding was confirmed by competition with cold, unlabeled PG but not with glycine-extended gastrin or amidated gastrin-17. Binding was not influenced by the presence of the classical CCK-2 receptor.

From these two studies, it was clear that a progastrin binding site/receptor exists, that is distinct from binding of amidated gastrin-17 and glycine extended gastrin-17. The sequence of progastrin that interacts with this receptor is probably in the COOH-terminal 26-amino acid residues of progastrin, shown to be sufficient for progastrin function (Ottewell et al., 2005), but the identity of this putative receptor is still an open question.

One candidate is Annexin A2, identified as being able to bind progastrin and derived peptides in 2006 by Singh et al. (Singh et al., 2006). Annexin A2 partially mediates the effect of progastrin/gastrins. In particular, Annexin A2 mediates up-regulation of NF-κB, β-catenin in response to progastrin in mice and HEK-293 cells (Sarkar et al., 2011). Also, Annexin A2 could be involved in progastrin endocytosis mediated by clathrins (Sarkar et al., 2011). However, the affinity of progastrin for annexin A2 is not what would be expected for a specific receptor. And, although Annexin A2 plays a role in progastrin functions, it is not that of a receptor.

Another candidate recently suggested is the G-protein coupled receptor 56 (GPCR56), expressed both on colonic stem and cancer cells (Jin et al., 2017). Indeed, while recombinant human progastrin promoted the growth and survival of wild-type colonic organoids *in vitro*, colonic organoids cultured from GPCR56-/- mice were resistant to progastrin. However, although it was shown that progastrin binds to GPCR56-expressing cells, authors did not provide the evidence of a direct binding to GPCR56 itself. GPCR56 is a good candidate, but the proof that it is THE progastrin receptor is still lacking.

The progastrin receptor can activate a number of signaling pathways, directly or indirectly, which is rather unusual for a receptor. This could indicate a particularity of that receptor, and the reason why it is difficult to identify.

The unidentified progastrin receptor transduces progastrin signal via various intracellular intermediates that are known for their involvement in tumorigenesis. ◆

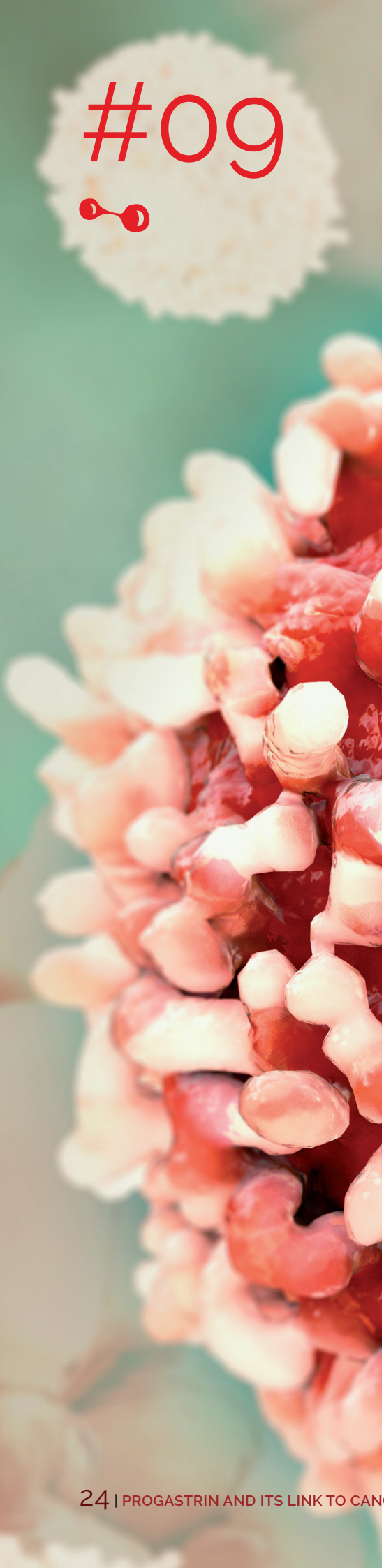
#08

PROGASTRIN AND ONCOGENIC SIGNALING PATHWAYS

The first demonstration of the link between progastrin and an oncogenic pathway was described for K-ras. Indeed, colon cancer cell lines and tissues with K-ras mutations all had significantly higher gastrin mRNA levels than those that were K-ras wild type (Nakata et al., 1998). The effects of K-ras on gastrin expression occurred through activation of the Raf-MEK-ERK signal transduction pathway, the final step being an activa-tion at the level of the gastrin promoter.

Oncogenic ras p60-Src, the first identified oncogene, is activated in colon cancer cells by increasing amounts of progastrin (Brown, 2003), meaning that the production of progastrin that occurs during early tumorigenesis (Pannequin et al., 2007), could play a role in this activation, which is known to be an early event in colon tumorigenesis (Cartwright et al., 1990; Iravani et al., 1998). PI3K/Akt, particularly involved in proliferation, is also activated by progastrin (Ferrand et al., 2005; Pannequin et al., 2007). Another major signaling messenger regulated by progastrin is NF-kappaB. Its involvement in the mechanisms responsible for the anti-apoptotic effect of progastrin have been demonstrated in pancreatic cancer cells *in vitro* (Rengifo-Cam et al., 2007) and *in vivo* in mice overexpressing progastrin (Umar et al., 2008). Up-regulation of Janus-activated kinase2, STAT3, and extracellular-signal regulated kinases has also been observed in the colonic mucosa of hGAS mice (Ferrand et al., 2005).

However, among all these regulations, the most important one is the link between progastrin and the Wnt pathway, which provides the essential understanding for progastrin to be considered today as a target to fight cancer.◆



#09

PROGASTRIN AND THE WNT PATHWAY

The Wnt pathway has been known for its involvement in tumorigenesis for many years, especially for cancer stem cell survival (Bhavanasi and Klein, 2016; Nusse and Clevers, 2017). In colorectal cancer, the Wnt pathway is constitutively activated in 80 to 90% of the tumors, with a somatic mutation in the APC gene in the majority of the cases. There are numerous genes whose expression is activated by the Wnt oncogenic pathway. The gene encoding progastrin is one of them. Indeed, Koh and colleagues have shown that the gastrin gene is a downstream target of the β-catenin/TCF-4 signaling pathway, and that transfection of a constitutively active ß-catenin expression construct causes a threefold increase in gastrin promoter activity (Koh et al., 2000). This is foundational work for the understanding of the link between progastrin and cancer because of the many cellular functions involving the Wnt pathway in a cancer cell, starting with its importance for cancer stem cell survival.

Since K-Ras and the Wnt pathways both induce progastrin gene expression, it was then hypothesized that there could be a cooperation between the two pathways in the regulation of progastrin expression. This is indeed what Chakladar and co-authors observed (Chakladar et al., 2005). They found a strong (25- to 40-fold) synergistic stimulation of the gastrin promoter by the combination of oncogenic ß-catenin and K-ras overexpression. Gastrin promoter activation could be further enhanced or suppressed by the co-expression of wild type SMAD4 or a dominant negative mutant of SMAD4, respectively, and abrogated by PI3K inhibition. Thus, the constitutive activation of the Wnt pathway, considered to be at the initiation of colon tumorigenesis, and oncogenic K-ras, present in 50 % of human colorectal tumors, stimulate synergistically progastrin production, a promoter of tumorigenesis. ◆

Since **K-Ras and the Wnt pathways both induce progastrin gene expression**, it was then hypothesized that there could be a cooperation between the two pathways in the regulation of progastrin expression.

#10

HOWEVER, AND OF PARAMOUNT IMPORTANCE: PROGASTRIN KNOCKDOWN CAN INACTIVATE THE WNT PATHWAY AND REVERSE TUMORIGENESIS

HOW WERE THESE CONLUSIONS DRAWN?

As described above, progastrin is a target gene of ß-catenin/Tcf4 transcription factors. But, does progastrin exert a feedback mechanism on this pathway and if yes, is it a positive or negative feedback loop?

The strategy to answer that question was simple: decrease progastrin production via siRNA and then meas-ure the transcriptional activity of ß-catenin/Tcf4 using a luciferase reporter assay. DLD-1 colorectal cancer cells were transfected, and the result showed that indeed, when progastrin production is impaired, ß-catenin/Tcf4 transcriptional activity is profoundly inhibited (Pannequin et al., 2007).

Thus, progastrin exerts a positive feedback on ß-catenin/Tcf4 activity. The mechanism of this feedback has been unraveled. It involves PI3K, ILK and ICAT. ICAT is an endogenous inhibitor of the ß-catenin-Tcf4 interaction. When expressed, ICAT binds ß-catenin, preventing its association with Tcf4. Both transcription factors de-localize to the cytoplasm, resulting in a *de facto* inactivation of the pathway (Pannequin et al., 2007).

The Wnt pathway, which is constitutively activated in colorectal cancer cells because of somatic mutation, can be inactivated, which was not thought to be possible at the time this work was performed. The consequence of this inactivation has been analyzed at different levels, including at the level of cell differentiation. Pannequin et al. demonstrated that the tumor cells that do not express progastrin return to a normal-like state. This is due to the fact that when the Wnt pathway is inactivated, a gene called jagged-1 is down regulated, which induces the inactivation of the Notch pathway that plays a major role in the acquisition of a differentiated phenotype (Pannequin et al., 2009). Cancer cells start to express the Muc2 gene, proof of their reacquired functional differentiation.

The consequence of the inactivation of the Wnt and Notch pathways by progastrin production inhibition was also observed in a mouse model that recapitulates intestinal tumorigenesis, the $APC^{\Delta 14/+}$ mouse model. These mice were either treated with siRNA (Pannequin et al., 2007) or with anti-progastrin antibodies (Prieur et al., 2017).

In both cases, the number of tumors that spontaneously develop in the intestine decreased, indicating that APC-driven tumorigenesis does in fact depend on progastrin.
The fact that inhibition of the Wnt pathway induces a "reversal" of tumorigenesis has been demonstrated by Dow et al. in 2015 (Dow et al., 2015). Authors of this work crossed mice harboring mutated K-Ras ($KRAS^{G12D}$) and P53 fl/fl with mice having an inducible shRNA APC. When APC was not expressed, Wnt activity was high, and tumors developed. When APC was expressed, Wnt activity was low, and tumors disappeared, despite the presence of $KRAS^{G12D}$ and P53 fl/fl.

This was a clear demonstration that targeting the Wnt pathway is sufficient to reverse tumorigenesis.

P53 is a tumor suppressor gene, whose absence is considered essential for tumor progression. In 2012, it was shown that P53 gene mutation increases progastrin dependent colonic proliferation and colon cancer formation in mice (Ramanathan et al., 2012). Progastrin is therefore a factor used by the cancer cell to sur-vive and evolve with time. The more the tumor progresses, the more it becomes dependent on progastrin. Targeting progastrin could therefore represent an efficient tool to fight cancer. ◆

In both cases, the number of tumors that spontaneously develop in the intestine decreased, indicating that **APC-driven tumorigenesis does in fact depend on progastrin**.

#11

PROGASTRIN AS A TARGET TO FIGHT CANCER

Until now, progastrin was not seen as a cancer target. The preceding data provides a strong basis for changing that perception: progastrin is found in the plasma of cancer patients and neutralization of progastrin induces tumor reversion.

Progastrin is indeed found in the plasma of cancer patients. Progastrin is detected from preneoplastic stages, such as adenomatous polyps. But as progastrin is produced by the tumor cells in the primary tumor and in the metastases, it is reasonable to propose that progastrin could be used for the follow-up of patients. It has been observed that circulating progastrin concentrations are increased in patients at risk of developing colorectal carcinoma (Paterson et al., 2014). It has also been observed that expression of progastrin in hyperplastic polyps was detected in the very few cases that evolved to a cancer (Do et al., 2012; Do and Seva, 2013). Furthermore, progastrin might also be a biomarker of liver metastasis in colorectal cancer (Westwood et al., 2017).

As far as progastrin is concerned as a therapeutic tool, the fact that cancer stem cells require progastrin to survive is fundamental since there are no drugs today able to target cancer stem cells (Prieur et al., 2017). Progastrin targeting also sensitizes tumor cells to radiotherapy, which could help radiotherapy be more efficient (Kowalski-Chauvel et al., 2017). In addition, chemotherapy induces a dramatic increase of progastrin in colorectal cancer cells, *in vitro* and *in vivo* (Prieur et al., 2017). This is in line with the fact that cancer stem cells escape chemotherapy, probably in part due to the production of progastrin that helps them survive. These observations indicate that a combination of chemotherapy (or any other anti-proliferative drug) with anti-progastrin antibodies could be very efficient in targeting both proliferating cells and cancer stem cells at the same time.

All the necessary data have been generated and published to support the rationale of progastrin as a new target in the fight against cancer.

The scientific community has acknowledged the role of progastrin in cancer development. It is now time that oncologists took a closer look at these data, and work with scientists to develop the tools that will be efficient for the patients in the fight against cancer. ◆

All the necessary data have been generated and published to support the rationale of progastrin as a new target in the fight against cancer.

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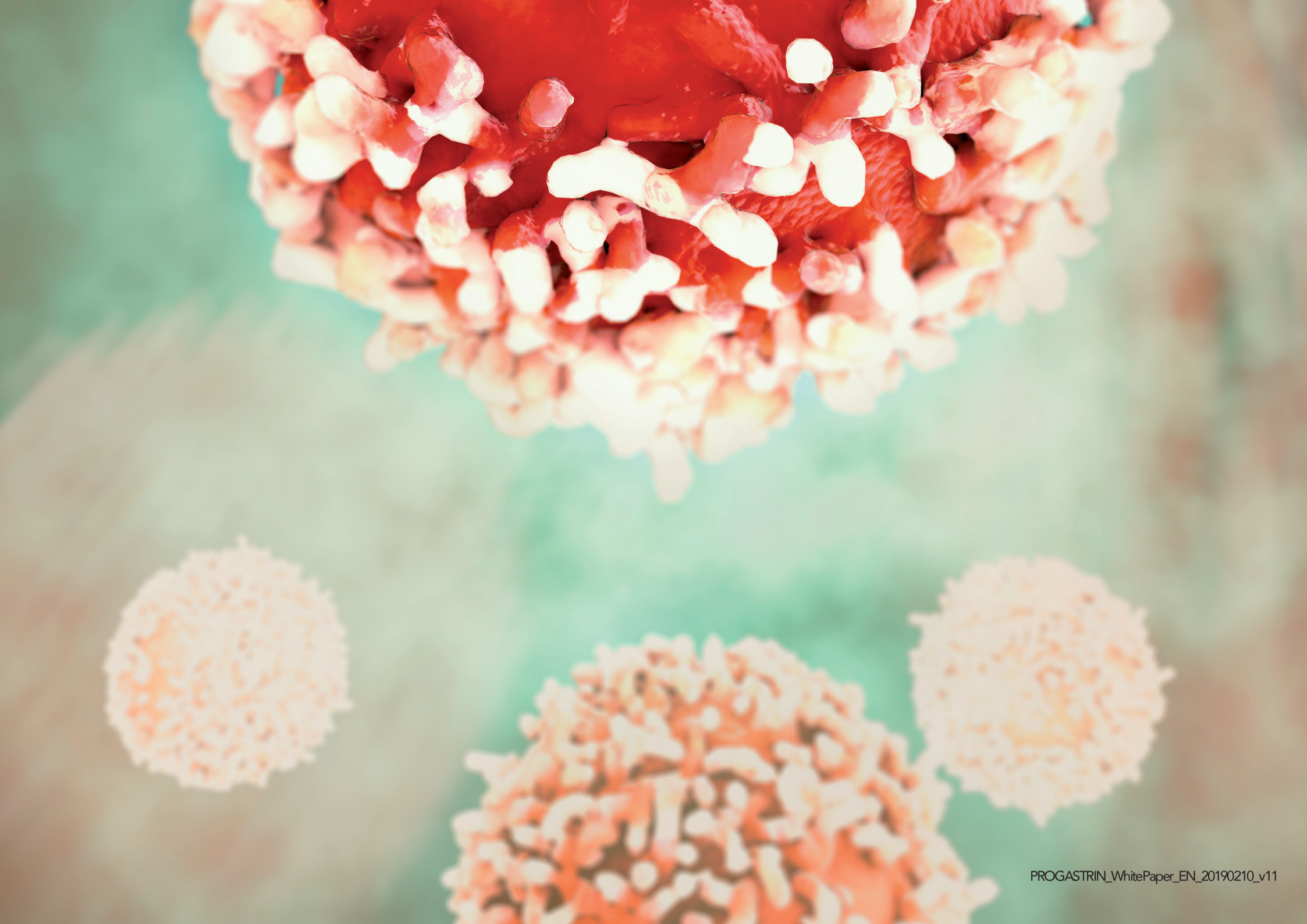

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